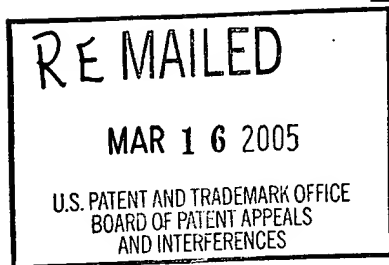


The opinion in support of the decision being entered today was not written for publication and is not binding precedent of the Board.

UNITED STATES PATENT AND TRADEMARK OFFICE

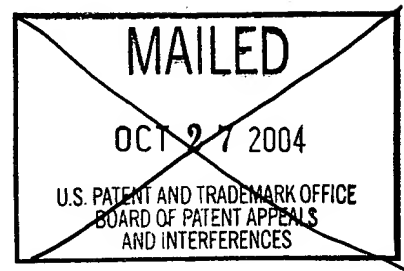
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Ex parte EUGEN KOREN and MIRNA KOSCEC



Appeal No. 2004-2138
Application No. 08/765,324

ON BRIEF



Before MILLS, GRIMES, and GREEN, Administrative Patent Judges.

GRIMES, Administrative Patent Judge.

DECISION ON APPEAL

This is a decision on appeal under 35 U.S.C. § 134 from the examiner's final rejection of claims 48-51, all of the claims remaining. Claims 48-51 read as follows:

48. A method for making antibodies to an epitope of a lipoprotein which reacts with the lipoprotein independently of lipid content and conformation of the lipoprotein, comprising

immunizing an animal with a desired apolipoprotein or lipoprotein which is delipidated, reduced, carboxymethylated, and solubilized with a reducing or denaturing agent, wherein all self-aggregated and degraded material has been removed from the delipidated, reduced, carboxymethylated, and solubilized apolipoprotein or lipoprotein.
49. The method of claim 48 further comprising

isolating the spleen from the immunized animals,

producing hybridomas from the spleen, and

screening the hybridomas for binding to the desired apolipoprotein or lipoprotein.

50. The method of claim 49 for making antibodies to a desired apolipoprotein wherein the apolipoprotein is selected from the group consisting of Apo A_I, Apo A_{II}, Apo B, Apo C_{III}, and Apo E.
51. The method of claim 49 for making antibodies to a lipoprotein wherein the lipoprotein is selected from the group consisting HDL, LDL, and VLDL.

The examiner relies on the following reference:

Lee et al. (Lee), "Properties of apolipoprotein B in urea and in aqueous buffers: The use of glutathione and nitrogen in its solubilization," Biochimica et Biophysica Acta, Vol. 666, pp. 133-146 (1981)

Claims 48-51 stand rejected under 35 U.S.C. § 112, first paragraph, as lacking an adequate written description in the specification.

We reverse and enter new grounds of rejection of the claims on appeal.

Background

Lipoproteins are classified according to their density; the classes of lipoproteins include very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL). Specification, page 1. Lipoproteins contain, among other things, proteins known as apolipoproteins. There are four groups of apolipoproteins: A, B, C, and E (or Apo A, Apo B, Apo C, and Apo E, respectively). Page 2. Each of these groups comprises at least two different proteins; for example, the Apo A group includes the proteins Apo A-I, Apo A-II, and Apo A-IV, and the Apo B group includes the proteins Apo B-100 and Apo B-48. Id.

Each of the classes of lipoproteins (LDL, HDL, etc.) in turn includes different apolipoproteins in different proportions. LDL includes only Apo B-100 as its protein component, although Apo B-100 is also present in VLDL and intermediate density lipoproteins (IDL). By contrast, approximately 90% of the apolipoprotein in HDL is Apo A-I or Apo A-II, while apolipoproteins in the Apo C and Apo E groups are present in all types of lipoprotein except LDL. Specification, page 2.

"Many epidemiological and clinical studies have shown that increased LDL levels in the blood are associated with increased risk of CHD [coronary heart disease]." Page 4. "In contrast . . . , individuals with high concentrations of HDL . . . seldom express symptoms of CHD." Page 5. The specification discloses "antibodies immunoreactive with specific epitopes on lipoproteins, such as those on LDL, VLDL and HDL, that enable rapid and reliable determinations of levels of lipoproteins and/or apolipoproteins in whole blood, serum or plasma." Page 14.

In particular, the specification discloses that "[c]onventional ways of producing MAbs [monoclonal antibodies] to Apo B-100 include immunization of mice with LDL. . . . However, MAbs produced using LDL as an immunogen tend to be sensitive to conformational changes of Apo B-100 caused by variations in the lipid composition of LDL particles." Page 26. "To obtain an anti-LDL MAb whose binding to LDL particles is not dependent on variations in LDL composition and/or conformation, mice were immunized with soluble Apo B-100 which had been delipidized, reduced, carboxymethylated and, purified by electrophoration in polyacrylamide gels containing 8 M urea (Lee, D.M. et al., Biochim. Biophys. Acta, 666:133-146 (1981))." Id., page 27.

"The spleen cells of mice that were immunized using the soluble and electrophoretically purified Apo B, were then used to produce hybridomas according to standard hybridoma methods." Id. One of the resulting monoclonal antibodies was designated HB₃cB₃. Id. "HB₃cB₃ binds to the epitope near the T2 carboxy terminal region of B-100, exclusively, and does not recognize B-48. The epitope recognized by HB₃cB₃ may be conformationally changed or masked by lipids and/or other apolipoproteins present in VLDL." Id. Thus, monoclonal antibody HB₃cB₃ binds exclusively to LDL.

Discussion

Claim 48, the only independent claim, is directed to a method for making antibodies that will react with a lipoprotein regardless of lipid content and conformation of the lipoprotein, by treating a lipoprotein or apolipoprotein to delipidate, reduce, carboxymethylate, and solubilize it with a reducing or denaturing agent, removing all self-aggregated and degraded material, and immunizing an animal with the treated apolipoprotein.

The examiner rejected claim 48, together with dependent claims 49-52, as containing new matter, i.e., lacking an adequate description in the specification. The examiner summarized her position as follows:

The entire written description support for these method claims [is] provided for on page 27, lines 5-16 (Example 2) and page 47, lines 15-34. . . . These passages do not provide for conception and written description support for that which is now broadly claimed because [they] do not provide conception by way of written description for (a) immunizing with lipoproteins or generic apolipoproteins so treated; (b) antibodies in general/polyclonal antibodies; (c) subgenus of reducing or denaturing agents; (d) immunization [with] soluble lipoprotein or apolipoprotein

produced by the method; and (e) generic means of removal of all self-aggregated and degraded material.

Examiner's Answer, page 6. The examiner further explained these points on pages 6-11 of the Answer.

Appellants argue that the specification's description satisfies 35 U.S.C. § 112, first paragraph:

The application has a long discussion of all of the various known apolipoproteins and which lipoproteins they form. The application describes how to specifically delipidated [sic], reduce, carboxylate [sic, carboxymethylate], and isolate antigen, as well as how to immunize animals, obtain polyclonal antibodies, and screen for the desired specificity. The application demonstrates how to make monoclonal antibodies, and recombinant antibodies with the same specificity. Nothing more is required.

Appeal Brief, page 8.

The examiner "'bears the initial burden . . . of presenting a prima facie case of unpatentability.' In re Oetiker, 977 F.2d 1443, 1445, 24 USPQ2d 1443, 1444 (Fed. Cir. 1992). Insofar as the written description requirement is concerned, that burden is discharged by 'presenting evidence or reasons why persons skilled in the art would not recognize in the disclosure a description of the invention defined by the claims.' . . . If . . . the specification contains a description of the claimed invention, albeit not in ipso verbis (in the identical words), then the examiner . . ., in order to meet the burden of proof, must provide reasons why one of ordinary skill in the art would not consider the description sufficient." In re Alton, 76 F.3d 1168, 1175, 37 USPQ2d 1578, 1583 (Fed. Cir. 1996).

"In order to satisfy the written description requirement, the disclosure as originally filed does not have to provide in haec verba support for the claimed subject matter at

issue." Purdue Pharma L.P. v. Faulding, Inc., 230 F.3d 1320, 1323, 56 USPQ2d 1481, 1483 (Fed. Cir. 2000). Nonetheless, the disclosure must convey with reasonable clarity to those skilled in the art that the inventor was in possession of the invention. See id.

The examiner set out five aspects of the claimed method that, in her opinion, were not adequately described in the specification. The examiner argues that the claims contain new matter because they are not limited to monoclonal (as opposed to polyclonal) antibodies; because they are not limited to the electrophoretic purification method disclosed in the specification; because they are not limited to the specific solubilization method used in the specification; because they read on immunizing an animal with a soluble lipoprotein, in addition to soluble apolipoprotein; and because the immunogen administered in the specification was not in soluble and reduced form because it was administered while still in a polyacrylamide gel (and therefore insoluble) and the specification does not disclose that the gel contained a reducing agent.

We agree with Appellants that none of the claim limitations pointed to by the examiner render the specification's description inadequate. We agree with Appellants' arguments that

[w]ith respect to . . . polyclonal antibodies, immunization of an animal with an antigen will always produce polyclonal antibodies. One must then isolate spleen cells and fuse these with immortal cells, which are then screened, for production of monoclonal antibodies.

With respect to the issue of "lipoprotein" versus "apolipoprotein", any one skilled in the art would understand that when one delipidates a lipoprotein, one by definition obtains an apolipoprotein. It is therefore irrelevant whether one starts with a lipoprotein or an apolipoprotein, one will utilize the same material as an antigen.

Appeal Brief, page 6. The examiner did not adequately rebut these arguments.

The examiner also objected to the claims' recitation of "solubiliz[ation] with a reducing or denaturing agent." See the Examiner's Answer, page 8: "The [relevant] passage [in the specification] does not specify how the ApoB-100 was solubilized and thus the amendment to provide solubilization with a reducing or denaturing agent provides a new subgenus of agents that is not supported by the original written description."

We do not agree with the examiner's reasoning. Whether a specification adequately describes a later-claimed invention is determined from the viewpoint of those of skill in the art. See, e.g., Eiselstein v. Frank, 52 F.3d 1035, 1039, 34 USPQ2d 1467, 1470 (Fed. Cir. 1995) ("The test is whether the disclosure of the application relied upon reasonably conveys to a person skilled in the art that the inventor had possession of the claimed subject matter at the time of the earlier filing date."). Here, the specification cites the Lee reference as the basis of the protocol used to solubilize Apo B-100. In addition, the examiner has provided no basis for concluding that those skilled in the art would not have been aware of reducing and denaturing agents, other than those used by Lee, that were commonly used to solubilize proteins. Thus, the examiner has not carried her burden of showing that the specification did not convey possession of this aspect of the method now claimed to a person of skill in the art.

The same is true of the examiner's concern regarding "generic means of removal of all self-aggregated and degraded material." Examiner's Answer, page 6. The specification describes purification by gel electrophoresis, Lee describes purification by gel filtration chromatography (page 136), and the examiner has provided no basis for concluding that those skilled in the art would not have been aware of other, equally

applicable methods of separating a reduced, carboxymethylated, and solubilized apolipoprotein away from self-aggregated and degraded material.

Finally, the examiner argues that the specification does not describe the claimed method because the Apo B-100 used as the immunogen in the specification was not soluble, since the protein was not removed from the polyacrylamide gel matrix before being injected into mice (and therefore was insoluble). We do not share this concern: both the specification (see page 27) and the Lee reference (see the abstract) make clear that those skilled in the art understood that the method steps recited in the claims produce "soluble" Apo B-100. That the soluble protein was then electrophoresed in a polyacrylamide gel does not change the soluble protein into an insoluble one; if the protein was removed from the gel, the skilled artisan would still expect the protein to be soluble in aqueous media. That is, those skilled in the art would understand the specification to describe a process of immunizing mice with a soluble protein, together with an insoluble polyacrylamide gel matrix.¹

We conclude that the examiner has not established that those skilled in the art would not recognize the specification's description to show possession of the method now claimed. The rejection under 35 U.S.C. § 112, first paragraph, is reversed.

New Grounds of Rejection

Under the provisions of 37 CFR § 41.50(b), we enter the following new grounds of rejection: claim 48 is rejected under 35 U.S.C. § 102(b) as anticipated by Lee, and

¹ Along the same line, the examiner argues that the Apo B-100 immunogen was not in a reduced form when it was injected, since the specification does not indicate that the polyacrylamide gel used to separate the intact Apo B-100 from self-aggregated and degraded material contained any reducing agent. This argument is addressed in the new grounds of rejection, below.

claims 49-51 are rejected under 35 U.S.C. § 103 as obvious in view of Lee and Goding.²

Lee teaches a method of making an antibody to LDL. Lee describes the preparation of the immunogen as follows: "The LDL₂ were delipidized wet with ethanol and diethyl ether, the latter being freed of peroxides before use. The LDL₂ apolipoprotein obtained was solubilized totally in 6 M guanidine HCl buffer containing the reducing agent dithiothreitol. After carboxymethylation, the reduced and carboxymethylated (RCM) LDL₂ apolipoprotein was purified by gel filtration to yield pure RCM apolipoprotein B." Abstract. The RCM apolipoprotein B was then used to immunize a rabbit (page 136, right-hand column).

Thus, Lee teaches a method of making antibodies comprising immunizing an animal with a desired apolipoprotein that has been delipidated, reduced, carboxymethylated, and solubilized with a reducing or denaturing agent (guanidine HCl buffer containing dithiothreitol), where all self-aggregated and degraded material have been removed from the treated apolipoprotein (by gel filtration). Thus, the method taught by Lee meets all the limitations of claim 48.

Lee does not teach a method of making monoclonal antibodies, as in instant claim 49. However, Goding teaches methods of making monoclonal antibodies to a desired antigen. It would have been obvious to a person of ordinary skill in the art at the time the invention was made to use the delipidated, reduced, carboxymethylated, and solubilized apolipoprotein taught by Lee as the antigen in Goding's method of making monoclonal antibodies. Motivation to combine the references is provided by Lee, which

² Goding, Monoclonal Antibodies: Principles and Practice, pp. 56-97, Academic Press, Inc. (1983).

teaches that the apolipoprotein that Lee used to raise antibodies is the major protein component of LDL, which is the principal carrier of cholesterol in the circulation (page 134, left-hand column); antibodies to the apolipoprotein would therefore have been expected to be useful in quantitating serum LDL levels.

Those skilled in the art would have been motivated to use Goding's methods of making monoclonal antibodies because such methods allow "a virtually unlimited supply of identical antibodies." Hybritech, Inc. v. Monoclonal Antibodies, Inc., 802 F.2d 1367, 1369, 231 USPQ 81, 82 (Fed. Cir. 1987). Thus, the method of claim 49 would have been obvious to those skilled in the art at the time of the invention. Lee's method involves making antibodies to Apo B, which will bind to LDL; the combination of Lee and Goding therefore meets all the limitations of claims 50 and 51.

The above rejections are basically the same as those made earlier in prosecution by the examiner. See the Office action mailed January 30, 2001. In response to these rejections, Appellants argued that Lee's method differs from the one claimed because Lee "does not immunize an animal with the delipidated, decarboxymethylated [sic], reduced apolipoprotein. He has removed the reducing agents from the apolipoprotein. In contrast, . . . applicants immunized with the delipidated, decarboxymethylated [sic], reduced apolipoprotein from which the degraded and complexed materials had been removed." Appellants' response to the Final Office action, received September 4, 2001.

The examiner withdrew the prior art-based rejections, but we believe they should have been maintained. The fact that Lee removed the reducing agent from the apolipoprotein preparation by dialyzing against distilled water is immaterial because the apolipoprotein had been modified by carboxymethylation after it was reduced. Lundblad

discusses carboxymethylation of proteins.³ See, e.g., Lundblad's Figure 2: treatment of a protein with a reducing agent like dithiothreitol breaks the disulfide bonds that normally exist between certain cysteine residues; thus, each $-S-S-$ bond becomes two $-SH$ groups (the starting point of the reaction in Lundblad's figure). Each $-SH$ group can then be carboxymethylated, converting it to an $-S-CH_2-COOH$ moiety.

Lundblad states that blocking the sulfhydryl ($-SH$) groups by, e.g., alkylation prevents them from reoxidizing to re-form disulfide bonds. See page 95. Thus, carboxymethylation prevents the protein from resuming its original, oxidized state; after carboxymethylation, the original $-S-S-$ bond cannot reform even if the reducing agent is removed.

For this reason, the fact that Lee removed the reducing agent from the reduced and carboxymethylated Apo B does not distinguish the method disclosed by Lee from the one claimed by Appellants. Both processes involve immunizing an animal with reduced, carboxymethylated, solubilized, and purified apolipoprotein. Lee therefore anticipates claim 48; combined with Goding, it would have made obvious claims 49-51.

Summary

The specification adequately describes the claimed process; Lee also describes the process of claim 48 and, combined with Goding, it would have made obvious the process of claims 49-51. We therefore reverse the examiner's rejection and enter two new grounds of rejection.

³ Lundblad et al., Chemical Reagents for Protein Modification, Volume I, pp. 55-60 and 95-98, CRC Press (1984), copy enclosed. We cite Lundblad only as evidence of how Lee would have been understood by those skilled in the art. Lundblad's disclosure is not necessary to reach any limitation of the claims on appeal.

Time Period for Response

This decision contains a new ground of rejection pursuant to 37 CFR § 41.50(b) (effective September 13, 2004, 69 Fed. Reg. 49960 (August 12, 2004), 1286 Off. Gaz. Pat. Office 21 (September 7, 2004)). 37 CFR § 41.50(b) provides "[a] new ground of rejection pursuant to this paragraph shall not be considered final for judicial review."

37 CFR § 41.50(b) also provides that the appellant, WITHIN TWO MONTHS FROM THE DATE OF THE DECISION, must exercise one of the following two options with respect to the new ground of rejection to avoid termination of the appeal as to the rejected claims:

(1) *Reopen prosecution.* Submit an appropriate amendment of the claims so rejected or new evidence relating to the claims so rejected, or both, and have the matter reconsidered by the examiner, in which event the proceeding will be remanded to the examiner. . . .

(2) *Request rehearing.* Request that the proceeding be reheard under § 41.52 by the Board upon the same record. . . .

REVERSED, 37 CFR § 41.50(b)


Demetra J. Mills
Administrative Patent Judge


Eric Grimes
Administrative Patent Judge


Lora M. Green
Administrative Patent Judge

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Notice of References Cited

Application/Control No.

08/765,324

Applicant(s)/Patent Under
Reexamination
Appeal No. 2004-2138

Examiner

BPAI

Art Unit

Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
	A	US-			
	B	US-			
	C	US-			
	D	US-			
	E	US-			
	F	US-			
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FOREIGN PATENT DOCUMENTS

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	O					
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	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
X	U	Lundblad et al., Chemical Reagents for Protein Modification, Volume I, pp.55-60 and 95-98, CRC Press (1984)
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Chemical Reagents for Protein Modification

Volume I

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THE MODIFICATION OF CYSTEINE

The sulfhydryl group of cysteine (Figure 1) is in general the most reactive functional group in a protein. Cysteinyl residues are easily alkylated, acylated, arylated, and oxidized (Table 1). The reactivity of cysteine is, as with most other functional groups in proteins, a reflection of the nucleophilic nature of the thiol groups. It is impossible to thoroughly discuss the reactions of protein sulfhydryl groups. The reader is directed to the excellent review by Liu¹ on the properties and reactions of sulfhydryl groups for a more extensive discussion of the chemistry of sulfur in proteins.

Cysteine is far more reactive as the thiolate anion. The pK_a for the formation of the thiolate anion is 10.5 with free cysteine but is considerably reduced with the cysteinyl residue in peptide bond. For example, the pK_a for the formation of the thiolate anion *N*-acetylcysteine ethyl ester is 8.5 while with *N*-formyl cysteine, it is 9.5. It is useful to compare these values with pK_a values for other functional groups as is done in Table 2.

The most useful class of reagents for the modification of cysteinyl residues in proteins has been the α -haloacetates and the corresponding amides. These reagents react with cysteine via a S_N2 reaction mechanism to give the corresponding carboxymethyl or carboxamidomethyl derivatives (see Figure 2). Any of the various α -halo acids or amides can be used. When a rapid reaction is desired, the iodine-containing compounds are used. For example, the reaction of iodoacetate with cysteine is approximately twice as fast as the reaction of bromoacetate and 20 to 100 times as rapid as chloroacetate. There are situations in which fast reaction rates are not necessarily desirable, such as the studies of Gerwin on streptococcal proteinase.² This particular study was of considerable importance since it emphasized the importance of microenvironmental effects on the reaction of cysteine with α -haloacids and α -haloamides. Chloroacetic acid was far less effective than chloroacetamide. The sulfhydryl group at the active site of streptococcal proteinase has enhanced reactivity in that modification with iodoacetate readily occurred in the presence of 100- to 1000-fold excess of β -mercaptoethanol or cysteine. The enhanced reactivity of the active site cysteine is also apparent from a comparison of the relative rates of modification of streptococcal proteinase and reduced glutathione. The rate of modification of streptococcal proteinase is 50 to 100 times more rapid than that of glutathione. The unique properties of this cysteine residue can be explained in part by the presence of an adjacent histidyl residue which was demonstrated by an elegant series of studies by Liu.³ Although histidine residues will react with α -halo acids and amides, the presence of an adjacent cysteine residue precluded the use of this class of reagents to demonstrate the presence of a histidyl residue at the active site of streptococcal proteinase. Liu took advantage of the reversible modification of cysteinyl residues with sodium tetrathionite⁴ to modify the active site histidine.

The reaction of chloroacetic acid and chloroacetamide with papain has also yielded interesting results.^{5,6} In studies with chloroacetamide, the active site sulfhydryl group of papain reacts at a rate more than tenfold faster than free cysteine ($5.78 \text{ M}^{-1} \text{ sec}^{-1}$ vs. $0.429 \text{ M}^{-1} \text{ sec}^{-1}$).⁵ As was the situation with streptococcal proteinase, there are dramatic differences in the rate of reaction of papain with chloroacetic acid and chloroacetamide. Figure 3 shows a first-order and second-order rate plot for the reaction of papain with chloroacetamide. It is of interest to note that the rate of inactivation continues to increase with increasing pH up to approximately pH 10.8 as shown in Figures 4 and 5. Note the difference in the observed behavior at different experimental conditions (here ionic strength was varied). The first-order and second-order rate plots for the reaction of papain with chloroacetic acid as reported by the same investigators are shown in Figure 6. This should be compared with Figure 3. The reaction with chloroacetic acid has a pH optimum of approximately 7 while the optimum

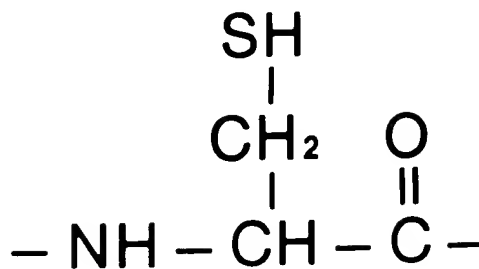


FIGURE 1. The chemical structure of covalently bound cysteine.

Table 1
REACTION OF PROTEIN FUNCTIONAL GROUPS

Amino acid	Alkylation	Acylation	Arylation	Oxidation
Methionine	x	—	x	x
Cysteine	x	x	x	x
Histidine	x	x	x	x
Lysine	x	x	—	—
Tyrosine	x	x	—	x
Tryptophan	—	—	—	x

Table 2
ACID DISSOCIATION
VALUES FOR
FUNCTIONAL GROUPS
IN PROTEINS

Functional Group	pKa
Carboxyl (Asp, Glu)	4.6
Imidazole (His)	7.0
Alpha-amino	7.8
Sulfhydryl (Cys)	8.5
Phenolic Hydroxyl (Tyr)	9.6
Side-chain amino (Lys)	10.5

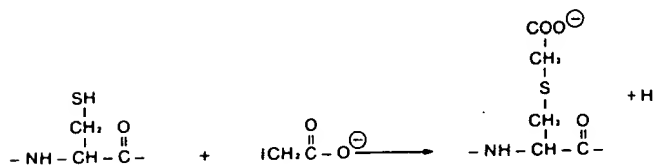


FIGURE 2. The modification of cysteine with iodoacetic acid to form S-carboxymethylcysteine.

for reaction with chloroacetamide is at a pH greater than 9. A comparison of the effect of pH on the reaction of papain with chloroacetic acid and chloroacetamide is shown in Figure 7. This investigation notes the influence of the neighboring histidyl residue as has been discussed for streptococcal proteinase. These data further emphasize the importance of neighboring functional group effects on cysteinyl reactivity in proteins as well as the im-

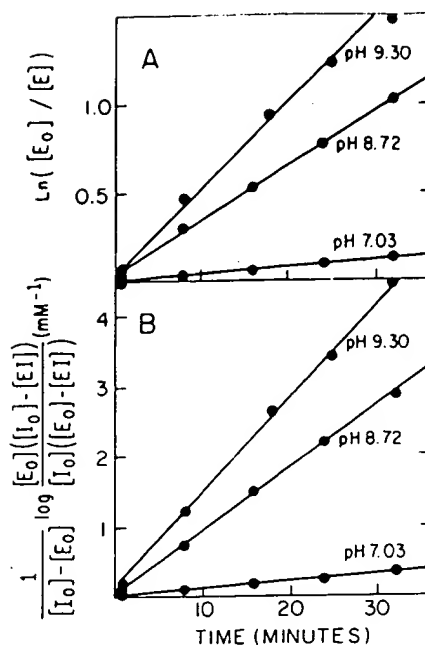


FIGURE 3. Rate plots for the inactivation of papain by chloroacetamide at various values of pH at low ionic strength (0.07). (From Chaiken, I. M. and Smith, E. L., *J. Biol. Chem.*, 244, 5087, 1969. With permission.)

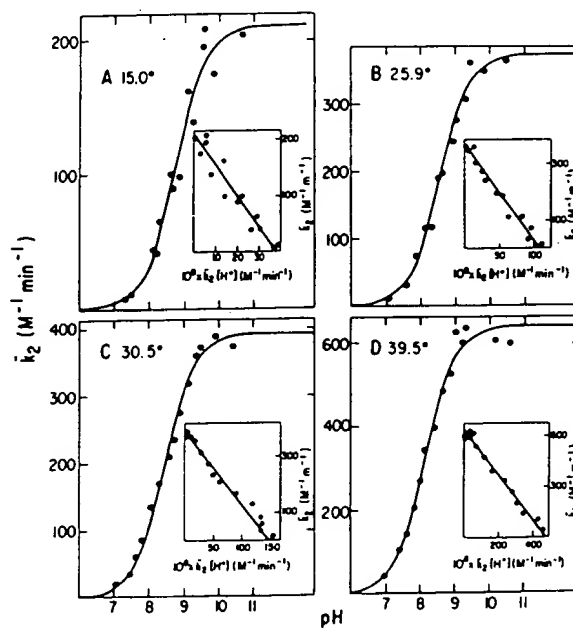


FIGURE 4. The effect of pH on the second order rate constant for the inactivation of papain by chloroacetamide at high ionic strength (0.50). (From Chaiken, I. M. and Smith, E. L., *J. Biol. Chem.*, 244, 5087, 1969. With permission.)

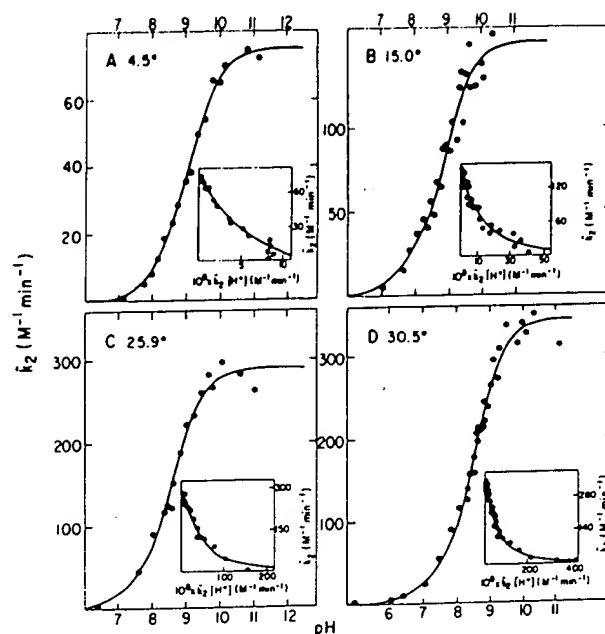


FIGURE 5. The effect of pH on the second-order rate constant for the inactivation of papain by chloroacetamide at low ionic strength (0.07). (From Chaiken, I. M. and Smith, E. L., *J. Biol. Chem.*, 244, 5087, 1969. With permission.)

portance of rigorous evaluation of the effect of pH on the rate of the modification reaction.

A complete consideration of all of the investigations which have utilized α -halo acids for the modification of sulfhydryl groups is beyond the scope of this limited work. This discussion will be confined to consideration of some of the most recent work with these derivatives as well as a consideration of some of the more unique reagents containing the α -halo, β -keto functions.

The α -halo acids decompose in water, with the rate being far more rapid at alkaline pH. In the case of iodoacetic acid, the products are iodide and glycolic acid. We recrystallize the commercially obtained reagents and store over P_2O_5 . The compounds are readily soluble in water. In the case of the free acid, it is useful to dissolve the compound in base prior to addition to the reaction mixture. In the case of α -haloacetyl derivatives, the resultant *S*-carboxymethyl cysteine is easily quantitated by amino acid analysis.

Jörmvall and co-workers⁷ have used reaction with iodoacetate to probe differences in structure in wild type β -galactosidase and various mutant forms of the enzyme. The modification reactions were performed in 0.1 M Tris, pH 8.1 under nitrogen in the dark. (This condition is of considerable importance since the α -halo acids are photolabile). The reaction was terminated by the addition of excess β -mercaptoethanol. Kalimi and Love⁸ have examined the reaction of the hepatic glucocorticoid-receptor with iodoacetamide in 0.010 M Tris-0.25 M sucrose. Again, this reaction was performed in the dark. Kallis and Holmgren⁹ have examined the differences in reactivity of two sulfhydryl groups present at the active site of thioredoxin. The pH dependence of the reaction with iodoacetate suggested that one group had a pKa value of 6.7 while the second was 9.0. Iodoacetamide showed the same pH dependence but the rate of reaction was approximately 20-fold greater than with iodoacetate. For example, at pH 7.2, the second order rate constant for reaction with iodoacetate was $5.2 M^{-1} sec^{-1}$ while it was $107.8 M^{-1} sec^{-1}$ for iodoacetamide. The results from this study are shown in Figure 8. The low pK of one of the sulfhydryl groups was suggested to be a

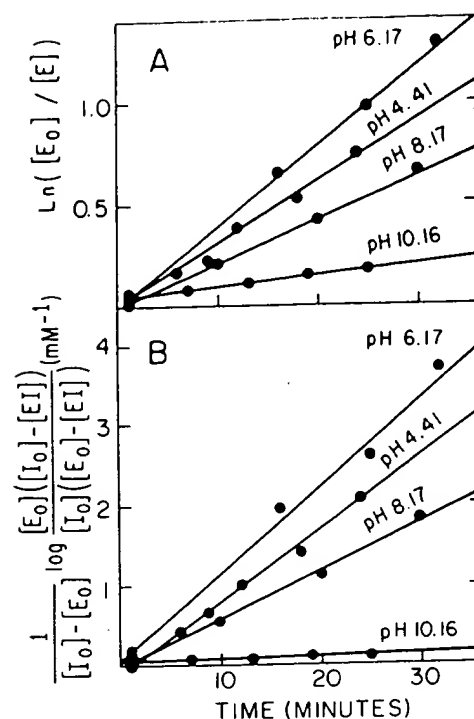


FIGURE 6. Rate plots for the inactivation of papain by chloroacetic acid at various values of pH at low ionic strength (0.07). (From Chaiken, I. M. and Smith, E. L., *J. Biol. Chem.*, 244, 5095, 1969. With permission.)

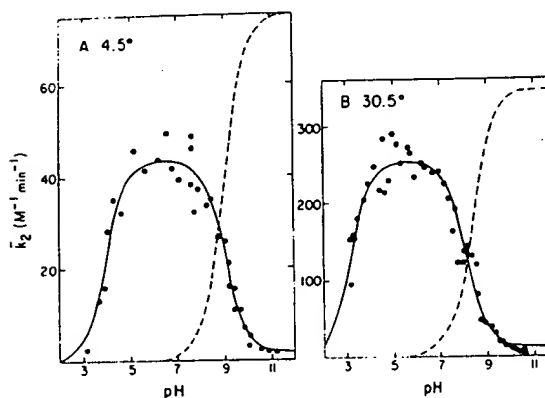


FIGURE 7. The effect of pH on the second-order rate constant for the inactivation of papain by chloroacetic acid at low ionic strength (0.07). The broken lines are the theoretical curves for the reaction of papain with chloroacetamide under the same reaction conditions. (From Chaiken, I. M. and Smith, E. L., *J. Biol. Chem.*, 244, 5095, 1969. With permission.)

reflection of the presence of an adjacent lysine residue. Mikami and co-workers have examined the inactivation of soybean β -amylase with iodoacetamide and iodoacetate.¹⁰ Inactivation with iodoacetamide occurred approximately 60 times more rapidly than with

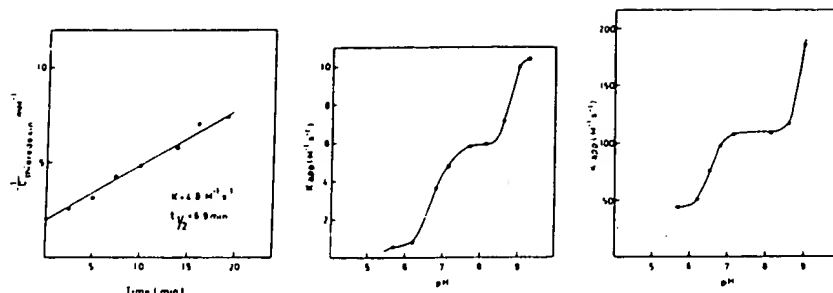


FIGURE 8. The left figure shows a time course for the reaction of thioredoxin and iodoacetic acid at pH 7.2. Analysis of this data yields a single second-order rate constant of $4.8 \text{ M}^{-1} \text{ sec}^{-1}$ and a halftime of 6.9 min. The center figure shows the effect of pH on the second-order rate constant for the reaction between iodoacetic acid and thioredoxin. The figure on the right shows the effect of pH on the second-order rate constant for the reaction between iodoacetamide and thioredoxin. (From Kallis, G.-B. and Holmgren, A., *J. Biol. Chem.*, 255, 10261, 1980. With permission.)

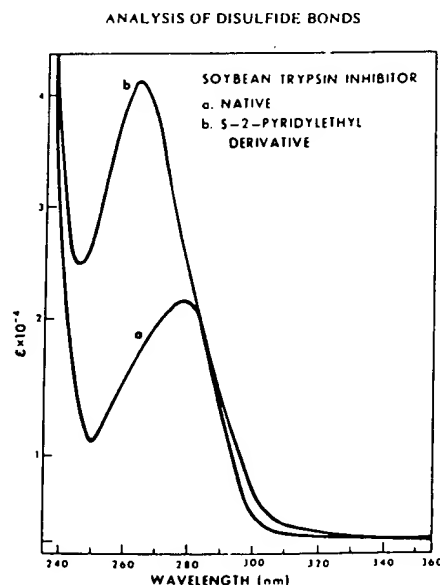


FIGURE 9. The UV absorption spectra of soybean trypsin inhibitor and the *S*-pyridylethylcysteinyl derivative of soybean trypsin inhibitor at a concentration of 0.5 mg/ml ($23 \mu\text{M}$) at pH 3.0 in 0.05 *M* glycine-HCl. (From Friedman, M., Krull, R. H., and Cavins, J. F., *J. Biol. Chem.*, 245, 3868, 1970. With permission.)

iodoacetate at pH 8.6. Hempel and Pietruszko¹¹ have shown that human liver alcohol dehydrogenase is inactivated by iodoacetamide but not by iodoacetic acid. These experiments were performed in 0.030 *M* sodium phosphate, pH 7.0 containing 0.001 *M* EDTA.

It should be noted that the reaction of sulfhydryl groups with iodoacetate¹² is still extensively used in the preparation of proteins for primary structure analysis, although pyridylethylation^{13,14} is proving to be quite useful (Figure 9).

A related compound which has proven useful is bromotrifluoroacetone.¹⁵⁻¹⁷ This derivative can be used to introduce ¹⁹F for nuclear magnetic resonance studies of the microenvironment surrounding the site of modification. This reaction is shown in Figure 10.

Chapter 7

THE MODIFICATION OF CYSTINE — CLEAVAGE OF DISULFIDE BONDS

It is generally accepted that disulfide bonds contribute substantially to the maintenance of the tertiary structure of single chain proteins such as ribonuclease^{1,2} as well as to maintain the structure of multi-chain proteins such as fibrinogen. The denaturation and cleavage of disulfide bonds are necessary for the enzymatic or chemical cleavage of peptide bonds for the production of fragments suitable for subsequent analysis.

There are also instances in which cleavage of specific disulfide bond(s) can provide useful information regarding the relationships between structure and function in a protein. Cleavage of the disulfide bond connecting the A and B chains in thrombin has been accomplished by Scheraga and co-workers³ in experiments which showed that the A chain did not have a critical role in the catalytic activity of this enzyme.

There are several approaches to the cleavage of disulfide bonds in proteins. The majority of studies involve the cleavage of the disulfide bond of cystine to the free thiol group of cysteine by reduction. Reduction has been generally accomplished with a mild reducing agent such as β -mercaptoethanol. Dithiothreitol has been a useful reagent in the reduction of disulfide bonds in proteins⁴ as introduced by Cleland. Dithiothreitol and the isomeric form, dithioerythritol, are each capable of the quantitative reduction of disulfide bonds in proteins. Furthermore, the oxidized form of dithiothreitol has an absorbance maximum at 283 nm ($\Delta\epsilon = 273$) which can be used to determine the extent of disulfide bond cleavage.⁵ The UV spectra of dithiothreitol and oxidized dithiothreitol are shown in Figure 1. Insolubilized dihydrolipoic acid has also been proposed for use in the quantitative reduction of disulfide bonds.⁶

In most proteins, the free sulfhydryl groups (cysteine) derived from the reduction of cystine will, at alkaline pH, fairly rapidly undergo reoxidation to form the original disulfide bonds. This process can be accelerated by the sulfhydryl-disulfide interchange enzyme^{2,7,8} or sulfhydryl oxidase.⁹ Thus, it is necessary to "block" the new sulfhydryl groups by alkylation, arylation or reaction with dithionite (see Chapter 6).

A novel reaction has been developed by Neumann and co-workers¹⁰ which allows for the reduction of disulfide bonds under mild conditions. Phosphorothioate reacts with disulfide bonds to yield the *S*-phosphorothioate derivatives.¹⁰ The reaction proceeds optimally at alkaline pH (pH optimum 9.7) and the reaction product, *S*-phosphorothioate cysteine, has an absorbance maximum at 250 nm ($\epsilon = 631 \text{ M}^{-1}\text{cm}^{-1}$) as shown in Figure 2. Phosphorothioate does not absorb at this wavelength. This reagent has been used to study the reactivity of disulfide bonds in ribonuclease.¹¹ In the absence of a denaturing agent (reaction conditions: tenfold molar excess of reagent, pH 9.0, 16 hr at 25°C), two specific disulfide bonds (Cys₆₅ - Cys₇₂; Cys₅₈ - Cys₁₁₀) are converted to phosphorothioate derivatives. The resultant derivative of ribonuclease is fully active in hydrolysis of RNA and has increased activity in the hydrolysis of cyclic cytidylic acid. The synthesis of radiolabeled phosphorothioate from either [³²P] or [³⁵S] thiophosphoryl chloride was reported in this study.

Light and co-workers have examined the susceptibility of disulfide bonds in trypsinogen to reduction.¹² At pH 9.0 (0.1 *M* sodium borate), a single disulfide bond (Cys₁₇₉ - Cys₂₀₃) is cleaved in trypsinogen by 0.1 *M* NaBH₄. The resulting sulfhydryl groups are "blocked" by alkylation. The characterization of the modified protein has been performed by the same group.¹³ The disulfide bond which is modified under these conditions is critical in establishing the structure of the primary specificity site in trypsin.

From the above studies, there is little doubt that the various disulfide bonds in a protein show different reactivity toward reducing agents. These differences in reactivity can be explored with various reagents and can be utilized with the aid of partial reduction followed

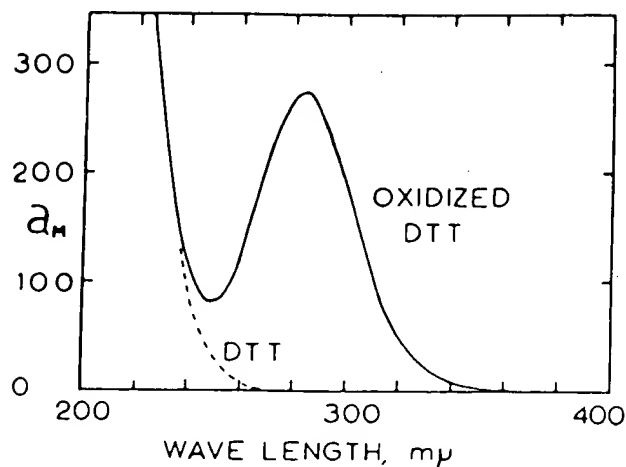


FIGURE 1. The absorption spectra of dithiothreitol (DTT) and oxidized dithiothreitol (oxidized DTT) in aqueous solution. (From Cleland, W. W., *Biochemistry*, 3, 480, 1964. With permission.)

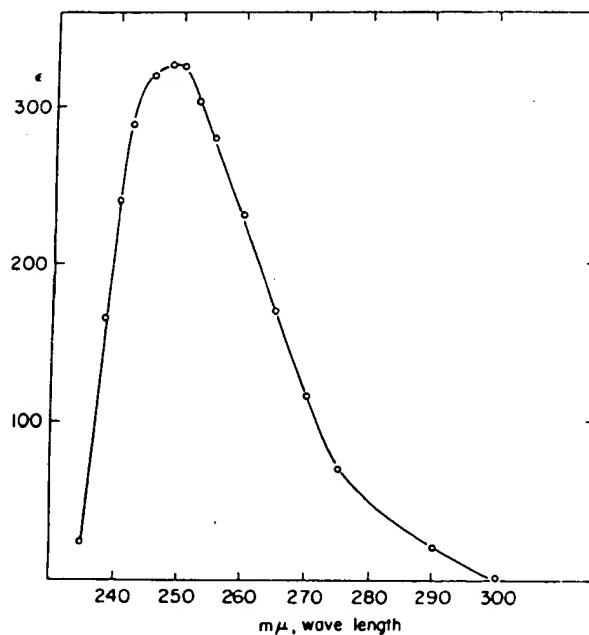


FIGURE 2. The absorption spectrum of the reaction product formed from cystine and phosphorothioate (PS). (From Neumann, H. and Smith, R. L., *Arch. Biochem. Biophys.*, 122, 354, 1967. With permission.)

by alkylation with radiolabeled iodoacetate to determine the position of disulfide bonds in proteins.¹⁴

Gorin and Godwin¹⁵ have reported that cystine can be quantitatively converted to cysteic acid by reaction with iodate in 0.1 to 1.0 M HCl. This reaction has been applied to insulin. The reaction product was not completely characterized, but given the relationship between iodate consumption and the cystine residues in insulin, the primary reaction is the oxidation

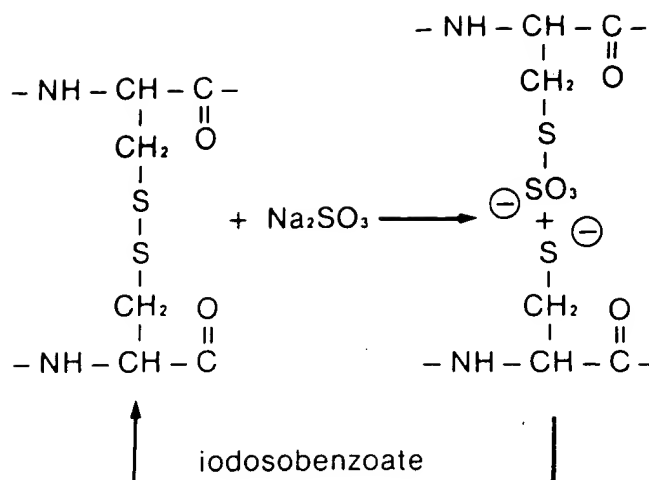


FIGURE 3. The cleavage of disulfide bonds by sodium sulfite to form the *S*-sulfo derivative.

of disulfide bonds. This reaction was complete in 15 to 30 min. After longer periods of reaction, the iodination of tyrosine residues occurred.

Disulfide bonds are somewhat unstable at alkaline pH ($\text{pH} \geq 13.0$). This has been examined by Donovan in some detail.¹⁶ With protein-bound cystine, there is change in the spectrum with an increase in absorbance at 300 nm. This problem has been more recently studied by Florence.¹⁷ This investigation presented evidence to suggest that cleavage of disulfide bonds in proteins by base proceeds via β -elimination to form dehydroalanine and a persulfide intermediate which can decompose to form several products.

The electrolytic reduction of proteins has been explored by Leach and co-workers.¹⁸ These investigators recognized that although small peptides containing disulfide bonds could be reduced using cathodic reduction, there would likely be problems with proteins because of size and tertiary structure considerations. Therefore, a small thiol was used as a catalyst for the reduction.

Gorin and co-workers¹⁹ have examined the rate of reaction of lysozyme with various thiols. At pH 10.0 (0.025 *M* borate), the relative rates of reaction were β -mercaptoethanol (2-mercaptoethanol), 0.2; dithiothreitol, 1.0; 3-mercaptopyruvate, 0.4; and 2-aminoethanethiol, 0.01. The results with 2-aminoethanethiol were somewhat surprising since the reaction (disulfide exchange) involves the thiolate anion and 2-aminoethanethiol would be more extensively ionized than the other mercaptans.

Finally, disulfide bonds can be cleaved by sulfite to form the *S*-sulfonate derivative as shown in Figure 3. The chemistry of this reaction has been reviewed by Cole.²⁰ The reaction proceeds optimally at alkaline pH (pH 9.0). It is necessary to include an oxidizing agent such as cupric ions, or as shown in Figure 3, *o*-iodosobenzoate to ensure effective conversion of all cystine residues to the corresponding *S*-sulfonate derivatives. The reaction is reversible to form cysteine upon treatment with a suitable mercaptan such as β -mercaptoethanol.

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